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PROJECTED USES OF CELLULAR MODELS AND FLUORESCENCE MICROSCOPY FOR IDENTIFICATION OF ANTIVESICANTS

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ABSTRACT

Although candidate antivesicant compounds have been tested on soldiers and other volunteers during wartime, animal models have been the basis for modern studies. Recent studies indicate that human skin cells can be used as in vitro subjects for testing of antivesicants. Fluorescent dye probes and a spectrofluorometer were used to observe effects of HD (sulfur mustard) in skin models. The Cytofluor 2300[®] revealed evidence of cell membrane damage in skin punch samples that were collected postmortem following sulfur mustard (HD) vapor challenges to hairless guinea pigs. This study is important because it suggests that dye probe readings can be used to correlate the results from parallel in vitro and in vivo investigations. This study also supports investigation of various potential fluorescent dye applications for measurement of other in vivo biochemical lesions. Possibilities for integration of the tripartite model/dye/Cytofluor system into the pre-existing antivesicant screening and development programs have been outlined.

INTRODUCTION

Sulfur mustard challenges have been delivered onto keratinized surfaces of commercially available artificial human skin and into culture medium of normal human epidermal keratinocytes (NHEK)^{1,2}. Testskin[®] (all product sources are listed in following sections) was provided on nutrient gel as thin wafers of sufficient diameter to support direct contact with 14 mm vapor cups configured for HD challenges of animals. Pre-production EpiDerm[®] (PreEpiD) was grown on microporous membranes within 12 mm Millicell CM[®] inserts. The PreEpiD was exposed to HD vapor within 10 mm vapor cups suspended from the rims of the Millicells. Undifferentiated NHEK on plastic culture plates were challenged with HD dissolved in culture medium. The various epidermal models have been incubated with fluorescent dye probes and examined in a spectrofluorometer. Such examinations revealed biochemical changes that are associated with microscopic changes³. After HD vapor cup exposures in vivo, vesicant damage has been observed by the methods of skin toxicology, microscopy and biochemistry⁴.

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BACKGROUND

One mission of the USAMRICD is to develop medical defenses against vesicant agents. This translates into objectives for development of products and methods that can be used under field conditions to defend military personnel from the effects of vesicants such as sulfur mustard (HD). Five types of possible products have been identified:

1. Topical skin protectant (TSP): barrier between skin and agent.
2. Decontaminant: to remove/detoxify agent at skin surface.
3. Pretreatment: to detoxify/prevent responses in skin or body.
4. Topical application: to limit post-exposure damage in skin.
5. Systemic treatment: whole body oral or parenteral medication.

Antivesicant development involves a multi-step screening program. Initial screening identifies unpromising candidates for elimination; intermediate screening tests are used to exclude less promising products; advanced testing is designed to select the best prospects for fielding. Such hierarchical programs (called decision trees by USAMRICD managers) must be implemented with suitable test models, methods and data to support critical decisions.

The hairless guinea pig is one model used to find possible defenses against HD damage in man, but alternatives are desired. Dr. Van Buskirk's proposal suggesting joint use of normal human epidermal keratinocytes (NHEK), fluorescent dye marker probes and spectrofluorometry led to a preliminary feasibility study. This showed that the Cytofluor 2300 (Millipore Corporation, Bedford, MA) can detect several different dye signals in non-chemically damaged NHEK cells of a human epidermal model. A second study revealed strong fluorescent dye signals from NHEK at 3 hr after damage from graded doses of the relatively weak vesicant 2-chloroethyl ethyl sulfide (CEES). The results of observations with inverted phase microscopy, scanning electron microscopy (SEM) and transmission electron microscopy have been reported. SEM showed structural changes, induced by CEES, in multilayered NHEK. Inverted phase contrast microscopy reveals dose-related widening of intercellular spaces with CEES and HD.

APPROACHES

Methods for toxicological studies involving fluorochrome dyes, living epidermal cells and an automated spectrofluorometer have been reviewed. These ideas have been considered for applicability to a pre-existing antivesicant development program. Five steps of this effort are as follows:

1. Select an animal model or other test model to represent some of the known human responses to vesicants and antivesicants.
2. Challenge the model with a range of vesicant concentrations or doses. This dose ranging exercise provides assurance that the reproducible dose-related and graded responses required for testing are obtainable with the model.
3. Measure normal responses of the model to the varied exposures.
4. Combine a candidate antivesicant treatment with agent exposure.
5. Choose candidates associated with maximal reduction of effects.

MODELS, METHODS AND MATERIALS

1. Epidermal cell models of human skin offer new possibilities for representation of known human responses to vesicants. The in vitro models of human skin that have been found usable with dye probe spectrofluorometry include the following.

a. Testskin[®] = Human Skin Equivalent (HSE)[®] (Organogenesis, Inc, Cambridge, MA). The manufacturer reports that this model is no longer on the open market. Formerly, Organogenesis cultured human fibroblasts with collagen to form Human Dermal Equivalent (HDE)[®] that was seeded with NHEK. NHEK basal cells were fed through HDE while stratum corneum formed on differentiated cells that were lifted into air with basal cells remaining in the culture medium.

b. PreEpiD, a pre-production skin model replaced by currently marketed EpiDerm[®] (MatTek, Corporation, Ashland, MA). Antecedent epidermal models¹ are formed from NHEK growing on cross-linked collagen gel adhered to the microporous membranes² of Millicell CM[®] inserts (Millipore Corp, Bedford, MA)³. Such inserts permit the differentiating NHEK to be fed initially from both top and bottom. Top feeding is stopped and NHEK are fed through the collagen gel while stratum corneum develops⁴. During studies of skin model feasibility for antivesicant testing, this pre-production version of EpiDerm, PreEpiD, was found to lack collagen⁵. The MatTek Corporation states that new EpiDerm, unlike PreEpiD, has substrate that is visible by light microscopy between basal cells and the microporous membrane of the insert⁶. EpiDerm's granular layer is also said to be more localized than in PreEpiD, being limited to 2 or 3 layers below the stratum corneum.

c. Confluent NHEK on Millicell inserts. This model was tried originally with dye probes and spectrofluorometry on the basis of published information⁷. The methodology was licensed to MatTek Corporation, which thereafter gel-coated government-furnished Millicells that were returned for NHEK seeding. This service was kindly provided under a Cooperative Research and Development Agreement. As used, NHEK formed more than one layer of cells^{8,9}.

d. Monolayers of NHEK on plastic. NHEK are cultured basal cells obtained (by Clonetics, Inc, San Diego, CA) after surgical circumcisions. Such cells multiply until they form a confluent layer (one cell thick) on the bottom surfaces of plastic wells that contain keratinocyte growth medium (KGM, provided by Clonetics with NHEK in an Epipak[®]).

2. Various vesicant challenges have been delivered to models by use of the following methods.

a. Testskin specimens were delivered as thin multicellular films (about 20 mm in diameter) that lay flat on a nutrient gel with stratum corneum uppermost. Vesicant vapor challenges were made by applying 12 mm (i.d.) vapor cups, as used on animals⁷.

b. PreEpiD specimens were located inside (and at the bottom of) 10 mm (i.d.) Millicell inserts. These were imbedded in nutrient gel held within the wells of culture plates in which the models were shipped. Vapor cups of 9 mm diameter (o.d.) were suspended inside of inserts to mimic exposures with 12 mm cups.

c. NHEK on plates or inserts were challenged with vesicant in 0.5 ml of MEM (minimum essential medium, Life Technologies, Inc, Grand Island, N.Y.). NHEK were on Falcon 3847 tissue culture plates (Becton Dickinson, Oxnard, CA). Final concentrations of CEES (or HD) were created in each of 24 wells by adding 10 μ l of ethanolic vesicant stock solution. An aliquot was immediately mixed with medium drawn into the disposable tip of a Pipetman[®] (Rainin Instrument Co, Woburn, MA). Alternately, challenges have been made by replacing nutrient medium with toxic medium that had been freshly thawed and mixed with HD to avoid hydrolysis of the HD¹⁰.

3. Normal responses of models to vesicant exposures have been seen or measured by use of methods described below.

a. For exploratory vesicant challenges, NHEK on inserts⁶ or culture plates¹ were exposed to 0, 0.8, 8.0 or 80.0 mM CEES (added as CEES in ethanol stock solution) for an hour under ambient conditions. NHEK were washed twice to remove toxic products and incubated with MEM until time for incubation with dye.

b. NHEK were incubated with dye in medium (usually 1:1000, v/v) for an hour, followed by triple washing to remove dye located external to the cell. All dye probes used in feasibility studies are designated by trade names of the vendor (Molecular Probes, Inc, Eugene OR). Calcein-AM is a non-fluorescent acetoxymethyl ester that is taken into cells and cleaved by intracellular esterases. It remains as a fluorescent marker until it leaks out through damaged cell membranes. Some cleaved dyes become fluorescent after binding Ca⁺⁺ (fluo-3), glutathione (CMFDA), or other biochemical target. Other dyes localize in cellular compartments such as lysosomes (neutral red) or mitochondria (rhodamine 123).

c. A Cytofluor 2300 spectrofluorometer was used to measure dye-induced fluorescence in washed cells. Cells or tissues in culture plates were illuminated with filtered UV light of appropriate wavelength to induce fluorescent signals. Signals were converted into numerical values by a computer for print out.

4. Combinations of challenges and candidate antivesicants include the following.

a. Incubation of candidates in media with cells before or after challenge. Pretreatment candidates are added before vesicant challenge; treatment candidates follow. TSP cytotoxicity can be monitored by applying TSP at the perimeter of the well, leaving a window of 8 mm diameter in the center for Cytofluor 2300 analysis.

b. TSP application over the stratum corneum of Testskin or PreEpiD prior to vesicant vapor challenge. PreEpiD is easier to use because cellular responses can be read from the bottom while TSP, decontaminant and agent products remain on top of the PreEpiD. Testskin required transfer of the fragile multilayered film from its bed of agarose gel to a 6-well culture plate for reading. Testskin would be difficult to use with liquid decontaminants.

c. Exposures of live animals defended with TSP, pretreatment, decontaminant, topical treatment or systemic antidote compounds. A method for collection of data from such exposures is described in Section 5.

5. Spectrofluorometry of skin from in vivo HD challenges:

a. Hairless guinea pigs were challenged by conventional methods with 7-min exposures to HD from vapor cups placed over 8 dorsal skin areas. These challenges were made by investigators to permit postmortem collection of 8-mm skin punch specimens for biochemical and histopathological examinations. Skin not used for histopathology was salvaged for spectrofluorometric examination. Skin punch discs were obtained from three animals at each of two post exposure time points: 5 hrs and 26 hrs. Two discs were punched from challenged contralateral sites of each excised skin specimen. Matched control discs were punched from the adjacent unexposed skin. Each disc was placed in warm (37°C) MEM for interlaboratory transport.

b. Curved surgical scissors were used to trim the muscle and subcutaneous tissue from skin discs. Each trimmed disc was placed into a well containing 1 ml of fresh warm MEM in a culture plate. The loaded plate was agitated for one minute at the 50% setting of a Minishaker (Creative Technologies, Greenwood Lake, NY). Each washed disc was blotted with paper toweling and placed into fresh warm MEM with 10 ug/ml of added calcein-AM. The loaded plate was incubated for one hour at 37°C in a 5% carbon dioxide atmosphere.

c. Incubated discs were washed and agitated twice with MEM by the method described in the above paragraph. Washed discs were placed in culture plate wells containing 0.5 ml of fresh warm MEM. Discs from the left sides of animals were placed with the stratum corneum facing the bottoms of the wells. Discs from right sides were placed with the trimmed dermal surface toward the bottoms. Loaded plates were taken into the Cytofluor 2300 for analysis according to published methods for analysis of keratinocytes^{2,3,5}.

RESULTS

Table 1 presents data collected postmortem from hairless guinea pigs given vapor cup exposures¹ by other investigators⁶ for data collection at 3 or 24 hr durations. Processing (as per para 5c, above) and transportation of specimens required approximately 2 hr. Statistical analysis shows dye loss (control reading greater than vapor-exposed reading, $p < .05$) in 3 of 4 test groups. These data are consistent with reported dye leakage from HD-damaged NHEK plasma membranes^{1,6,7,8}. No difference is observed between readings via stratum corneum (to eliminate trimming errors) and readings via trimmed dermis. Results suggest that additional trials should be made with other fluorescent dye probes, readings in other time frames and after other Ct (concentration x time) vapor doses.

TABLE 1. CYTOFLUOR READINGS FROM CALCEIN IN PUNCHED ANIMAL SKIN

5 hr after 7 min HD vapor				26 hr after 7 min HD vapor			
I.D.	Control	Vapor	% Loss	I.D.	Control	Vapor	% Loss
1S	1542	1107	28.2	4S	2944	2411	18.1
2S	1555	1191	23.4	5S	2775	2060	25.8
3S	1520	1288	15.3	6S	2003	1194	40.4
1D	3446	2594	24.7	4D	4526	2969	34.4
2D	1466	1363	07.0 n.s.	5D	3676	2473	32.7
3D	1856	1449	21.9	6D	2927	2137	27.0

S: Stratum corneum up, reading through dermis. D: Dermis up. n=3.
n.s.: Control vs vapor difference not significant for this group.

* Statistical analysis by Ms Robyn Lee.

DISCUSSION OF POTENTIAL USES OF NEW METHODS

Further extensions of the described methodology are being considered. These are outlined below:

1. It appears that intercellular spacing of cells on plastic could be measured by image analysis for screening purposes. The relationships of alkylation (by vesicants) and adhesion complexes and/or ionic binding should be investigated.

2. Confocal microscopy should permit localization of probes to reveal sites at which Ca^{++} or glutathione releases occur and may track other probes to reveal different kinds of damage. Confocal observations may explain toxic mechanisms, suggest new antivesicant candidates and support new approaches.

3. It should be possible to correlate fluorescence data from whole animals with data from in vitro assemblies of defined cell types. For example, NHEK on inserts could chemotactically attract WBCs from medium in wells plated with endothelial cells or other mediator sources. T-lymphocytes or other special cells could be co-challenged with NHEK, added separately or added at calculated time intervals. Endothelial cells could exist in Millicell inserts over NHEK on plastic, or vice versa. Cell types could be labelled with separate dye probes, radiolabels or specific labeled antigens. These or other approaches with combined cell types may facilitate factoring of cytotoxic and inflammatory reactions.

4. A hand held or enclosed Cytofluor sensor may permit in vivo readings of responses in animals given dye probes subcutaneously. Laser light sources may improve sensitivity of dyes in skin/cells.

5. It has been suggested¹¹ that biochemical changes in medium around challenged cells and in skin should be tracked with improved microdialysis probes¹² and microanalysis methods.

6. Progress now being made in manufacture, storage, shipping, use, and fluorescence probing of cellular models can be expected to improve the feasibilities and economics of described and new tests. There is hope that cells can be maintained in a state of suspended activity for shipment or for synchronization of experiments.

7. Parallel observations of changes in cells (Cytofluor), of cells (structure), around cells (microdialysis - of cultures or of skin in vivo) and in animals (punch analysis, histology, signs) are now conceivable. Analyses of such observations may elucidate the relative contributions of vesicants to membrane and cytoplasmic cytotoxicity, inflammatory processes, nutrient losses, immunologic mechanisms, repair systems and reproductive damage.

8. Metabolic or toxic effects of antivesicants, and specific protective effects, should be discernible with use of fluorescent labels. For example, use of cell membrane labels and inverted fluorescence microscopy or confocal microscopy should permit visualization of cellular attachments, antibody binding or lack thereof, and observation of protease activities. Furthermore, some proteases can be identified and quantified by using specific fluorescent substrates.

9. Protection by a drug has been identified and explained¹³, as antivesicant protection might be, with the above described system. Preliminary observations of multiple end point responses of NHEK challenged with CEES have stimulated attempts to elucidate mechanisms of vesicant toxicity involving Ca^{++} fluxes and other effects observed with the tripartite system¹⁴.

CONCLUSIONS

1. In vitro human skin cells and epidermal models can be used to observe vesicant agent effects on skin structure and intracellular biochemistry.
2. The tripartite system for use of human skin cells, fluorescent dye probes and spectrofluorometry can be used to screen and develop antivesicants, perform safety evaluations and study toxicologic mechanisms of vesicants.
3. The dye probe/spectrofluorometry method has demonstrated plasma membrane damage in excised skin from animals exposed in vivo to HD vapor doses that have been associated with marked erythema. Skin biopsies may be expected to provide similar results.
4. It should be possible to design in vitro and in vivo experiments for multiple-end-point observations with fluorescent dye probes and spectrofluorometry in parallel with the use of microdialysis tubes to collect biochemicals from challenged cells and tissues.
5. The described methods have potential for extension in the development of new methods.

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